Troglitazone inhibits angiotensin II-induced extracellular signal-regulated kinase 1/2 nuclear translocation and activation in vascular smooth muscle cells

Stephan Goetze[a,b], Xiao-Ping Xi[a], Kristof Graf[b], Eckart Fleck[b], Willa A. Hsueh[a], Ronald E. Law[a,*]

[a] *University of California, Los Angeles, School of Medicine, Division of Endocrinology, Diabetes and Hypertension, Warren Hall, Second Floor, Suite 24-130, 900 Veteran Avenue, Box 957073, Los Angeles, CA 90095, USA*
[b] *Department of Medicine/Cardiology, Virchow Klinikum, Humboldt University Berlin and German Heart Institute Berlin, 13353 Berlin, Germany*

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Abstract **The thiazolidinedione troglitazone inhibits angiotensin II-induced extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase activity in vascular smooth muscle cells. Activation of extracellular signal-regulated kinase 1/2 by angiotensin II is a multistep process involving both its phosphorylation by mitogen-activated protein kinase extracellular signal-regulated kinase kinase in the cytoplasm and a subsequent translocation to the nucleus. The cytoplasmic activation of extracellular signal-regulated kinase 1/2 in vascular smooth muscle cells proceeds through the protein kinase Cζ → mitogen-activated protein kinase extracellular signal-regulated kinase kinase → extracellular signal-regulated kinase pathway. Troglitazone did not affect the angiotensin II-induced activation of protein kinase Cζ or its downstream signaling kinases extracellular signal-regulated kinase 1/2 in the cytosol. In contrast, angiotensin II-induced activation of protein kinase Cζ and extracellular signal-regulated kinase 1/2 in the nucleus were both inhibited by troglitazone. Nuclear translocation of extracellular signal-regulated kinase 1/2 induced by angiotensin II was completely blocked by troglitazone. Protein kinase Cζ, however, did not translocate upon angiotensin II stimulation. Troglitazone, therefore, inhibits both angiotensin II-induced nuclear translocation of extracellular signal-regulated kinase 1/2 and the nuclear activity of its upstream signaling kinase protein kinase Cζ. Since extracellular signal-regulated kinase 1/2 nuclear translocation may be a critical signaling step for multiple growth factors that stimulate vascular smooth muscle cells proliferation and migration, troglitazone may provide a new therapeutical approach for the prevention and treatment of atherosclerosis and restenosis.**

Key words: Extracellular signal-regulated kinase 1/2; Protein kinase Cζ; Vascular smooth muscle cell; Troglitazone; Angiotensin II

1. Introduction

Angiotensin II (AII) is an important regulator of multiple vascular functions and contributes under pathological conditions to alterations in the arterial wall by stimulating vascular smooth muscle cell (VSMC) proliferation and migration [1,2]. These effects of AII on VSMC are extracellular signal-regulated kinase (ERK) 1/2-dependent and mediated via the AT-1 receptor [3,4]. Previously, we demonstrated that the thiazolidinedione troglitazone (TRO) blocked AII-induced DNA synthesis and migration in VSMCs by inhibiting ERK 1/2 activation [5]. TRO also inhibited bFGF-induced proliferation and PDGF-directed migration through ERK 1/2-dependent pathways [6]. In contrast to its action on the AII signaling pathway, TRO did not affect PDGF- or bFGF-induced ERK 1/2 activity [6], suggesting that TRO targets two or more intracellular sites, which all ultimately result in a suppression of ERK 1/2 signaling.

In VSMCs, signaling to ERK 1/2 by AII is mediated via its G-protein-coupled seven domain transmembrane AT1 receptor [7] while bFGF and PDGF induce signal transduction through their tyrosine kinase receptors [8]. Activation of ERK 1/2 in response to bFGF and PDGF is mediated through the ras/raf/mitogen-activated protein kinase (MAPK) ERK kinase (MEK)/ERK 1/2 signaling cascade [9,10], whereas recent studies report that the atypical protein kinase Cζ (PKCζ) can function as an upstream serine/threonine kinase for AII-induced MEK and ERK 1/2 activation in VSMCs [11]. In addition, several studies describe a nuclear translocation of PKCζ after growth factor stimulation [12–14], where it phosphorylates intranuclear targets such as nucleolin and potentially ERK 1/2 via MEK [15].

ERK 1/2 also translocates into the cell nucleus after growth factor stimulation [16,17], where it phosphorylates and activates multiple transcription factors which modulate gene expression to regulate cell proliferation [18,19]. AII is known to induce a prominent translocation of activated ERK 1/2 into the cell nucleus of neuronal cells, where ERK 1/2 functions to mediate nuclear events such as activation of the serum response element (SRE), c-fos expression and an increased AP1-binding activity [20]. Recent studies reporting the inhibition of VSMC migration by the p21 cyclin-dependent kinase inhibitor (Cip 1) and cyclin G1 antisense constructs suggest that nuclear events are also required for migration [21,22]. This is in line with our finding that migration of VSMCs towards multiple growth factors is blocked by transcriptional inhibition with actinomycin D [23].

TRO is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ) [24], a ligand-activated transcription factor of the steroid hormone nuclear receptor

*Corresponding author. Fax: (1) (310) 794 7654.
E-mail: rlaw@med1.medsch.ucla.edu

Abbreviations: TRO, troglitazone; VSMC, vascular smooth muscle cell; AII, angiotensin II; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK ERK kinase; PKCζ, protein kinase Cζ; PPARγ, peroxisome proliferator-activated receptor γ; MBP, myelin basic protein

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superfamily [25]. An important objective of this study was to determine whether TRO exerts its inhibitory effects on AII-induced signaling at a nuclear level or merely targets cytosolic signaling events. To elucidate which steps in the ERK 1/2 signaling cascade are affected, we investigated the effects of TRO on AII-induced activation of PKCζ and ERK 1/2 in the cytosolic and nuclear cell compartments.

2. Materials and methods

2.1. Materials

Materials were obtained from the following suppliers: Dulbecco's modified Eagle's medium (DMEM), glutamine, antibiotics, HEPES, DMSO, myelin basic protein (MBP) and monoclonal antibody against smooth muscle α-actin from Sigma (St. Louis, MO, USA), AII was from Bachem (Torrance, CA, USA), [γ-^{32}P]ATP was from ICN (Irvine, CA, USA), Hybond ECL nitrocellulose membrane, horseradish peroxidase-linked anti-rabbit antibody, as well as ECL Western blotting detection reagents were from Amersham Life Sciences (Arlington Heights, IL, USA). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA, USA). Sprague-Dawley rats were from Charles River (MA, USA). The phospho-specific- and total ERK1/ERK2 ERK 1/2 rabbit antibodies were purchased from New England BioLabs (Beverly, MA, USA). TRO was kindly provided by Parke Davis (Ann Arbor, MI, USA). Antibodies against PKCζ were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.2. Cell culture

Rat aortic smooth muscle cells were prepared from thoracic aortas of 2–3 month old Sprague-Dawley rats using the explant technique [26]. The cells were cultured in DMEM containing 10% FBS, 150 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 mmol/l glutamine. A monoclonal antibody against smooth muscle α-actin was used to assess the purity of the smooth muscle cell cultures. Flow cytometry of anti-smooth muscle α-actin antibody-stained cells revealed a purity of 95 ± 3%. For all experiments, early passaged (five or less) VSMCs were grown to 60–70% confluency and made quiescent by serum starvation (0.4% FBS/DMEM) for at least 24 h. When used, TRO was added 30 min prior to the addition of AII. For all data shown, each individual experiment represented in the *n* value was performed using an independent preparation of VSMCs.

2.3. Western blot analysis

For protein analysis, cultured VSMCs were grown to 60–70% confluency and then starved for 24 h in 0.4% FBS/DMEM. For inhibitor studies, cells were pre-treated for 30 min with TRO (10 μM) or vehicle (0.4% FBS/DMEM) alone, followed by the addition of AII (1 μM). Cells were then washed twice with ice-cold PBS and lysed with radioimmunoprecipitation (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% NP 40, 150 mM NaCl, 2.5 mM Na-pyrophosphate, 100 μM Na-orthovanadate, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 10 mg/l leupeptin) for 20 min on ice. After allowing the cells to thaw, they were scraped off the dish and centrifuged (14 000 rpm, 30 min at 4°C). For some experiments, nuclear and cytosolic fractions were prepared by the method of Dignam et al. [27]. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, San Diego, CA, USA). Equal amounts of protein (30 μg) were then separated by SDS-PAGE (7.5% standard gel) and transferred to nitrocellulose membranes using a Bio-Rad transblotter. Non-specific binding was blocked by using 5% fat-free milk powder and 0.1% Tween 20 in TBS. The membranes were incubated with rabbit polyclonal antibodies that recognize ERK1 or ERK2 which are phosphorylated on threonine 202 and tyrosine 204, i.e. 'phospho ERK/ERK2', or PKCζ protein. Antibodies were used at a concentration of 1:1000 for 1 h in blocking solution. Blots were washed three times for 15 min in 0.1% Tween 20/TBS, incubated for another hour with goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:1000) for 1 h and washed again before final development using the ECL Western blotting detection system. All Western blot experiments were repeated at least three times with a different cell preparation.

2.4. PKCζ activity measurements

To measure PKCζ activity, VSMCs were lysed in buffer A (2.5 mM Na-pyrophosphate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 1 mM PMSF, 20 mM Tris, pH 7.5, 1% Triton X-100, 1 mM β-glycerolphosphate, 1 mM Na-orthovanadate) and then flash frozen on a dry ice-ethanol bath. After allowing the cells to thaw, cells were scraped off the dish, centrifuged at 14 000 rpm (4°C for 30 min) and protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Richmond, VA, USA). Equal amounts of proteins (50 μg) in 100 μl buffer A were incubated overnight with rabbit polyclonal antibodies (1:50) against PKCζ at 4°C. Then, 40 μl protein Sepharose G beads (Pharmacia Biotech, Piscataway, NJ, USA) was added to each sample, followed by incubation for 2 h at 4°C. After that, the samples were centrifuged at 14 000 rpm for 30 s, pellets were washed twice with buffer A and then twice with buffer B (25 mM Tris-HCl, pH 7.5, 0.5 M LiCl) and once in 25 mM Tris-HCl, pH 7.5. Beads were resuspended in 15 μl kinase buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 25 μM ATP) to which 5 μg MBP (21 kDa) and [γ-^{32}P]ATP (5 μCi) was added. Samples were incubated for 30 min at room temperature. Then, the kinase reaction was stopped by boiling samples for 5 min in Laemmli sample buffer. Protein samples were then separated by SDS-PAGE (12% standard gel), the gel was dried and then subjected to autoradiography.

2.5. Statistics

Analysis of variance and paired or unpaired *t* tests were performed for statistical analysis, as appropriate. *P* values less than 0.05 were considered to be statistically significant. Data are expressed as mean ± S.E.M.

3. Results

3.1. AII-induced activation of cytosolic PKCζ is not affected by TRO

We have previously shown that TRO inhibits AII-induced

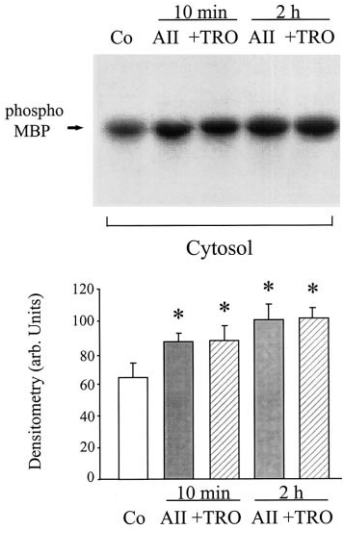

Fig. 1. TRO does not affect the AII-induced cytosolic PKCζ activity. Quiescent VSMCs were incubated with TRO (10 μM) for 30 min before AII (1 μM) was added. After stimulation with AII for 10 min or 2 h, cytosolic cell extracts were prepared and equal amounts of protein (50 μg) were immunoprecipitated with a PKCζ antibody. Kinase assays for PKCζ activity were then performed as described in Section 2 and samples were separated on 12% SDS-PAGE gels. Phosphorylation of MBP (phospho-MBP) by PKCζ was determined by autoradiography. The autoradiogram shown is representative of three experiments using different cell preparations. Densitometric analysis was performed using the NIH Image program 1.60 for Macintosh. The data were calculated in arbitrary units and are expressed as mean ± S.E.M., * = $P < 0.05$ versus control.

ERK 1/2 activation in VSMCs [5]. Since PKCζ functions as an upstream kinase that activates and phosphorylates MEK [11,28,29], which in turn phosphorylates and activates ERK 1/2, we examined the effect of TRO on AII-induced PKCζ activity in the cytosol. Using an immune complex phosphorylation assay, we observed a constitutive PKCζ activity in cytosolic extracts from untreated controls, which was significantly increased after stimulation with AII at 10 min and 2 h (by 1.45-fold and 1.62-fold over control, $P < 0.05$). Treatment with TRO had no effect on the basal or AII-induced cytosolic PKCζ activity (Fig. 1).

3.2. TRO inhibits the nuclear ERK 1/2 activity, but not its cytosolic activation by AII

Our previous results on TRO's inhibition of AII-induced ERK 1/2 activation were obtained in whole cell extracts [5]. Studies in other cell types, however, have demonstrated that AII can differentially regulate the ERK 1/2 activity in cytosolic and nuclear compartments due to its stimulation of ERK 1/2 translocation to the nucleus [20]. We therefore next examined the effect of TRO on cytosolic or nuclear AII-induced ERK 1/2 activation.

AII induced a rapid and transient activation of cytosolic ERK 1/2 at 10 min (by a 6.2 ± 0.6-fold over control, $P < 0.05$) with a return to baseline levels at 2 h (Fig. 2). TRO had no effect on the activation of cytosolic ERK 1/2 by AII. In contrast, AII stimulated a marked increase in activated nuclear ERK 1/2 at 10 min (16-fold) and 2 h (5.7-fold) which was dramatically inhibited by TRO (inhibition by 92 and 65%, respectively), indicating that TRO exerts its inhibitory effects on ERK 1/2 signaling at a nuclear level.

3.3. TRO inhibits AII-induced nuclear translocation of ERK 1/2 in VSMCs

Inhibition of AII-stimulated nuclear ERK 1/2 activity by TRO could result either indirectly by preventing the nuclear translocation of activated ERK 1/2 or directly by inhibiting the activation of ERK 1/2 residing in the nucleus. We therefore investigated whether AII induces nuclear translocation of

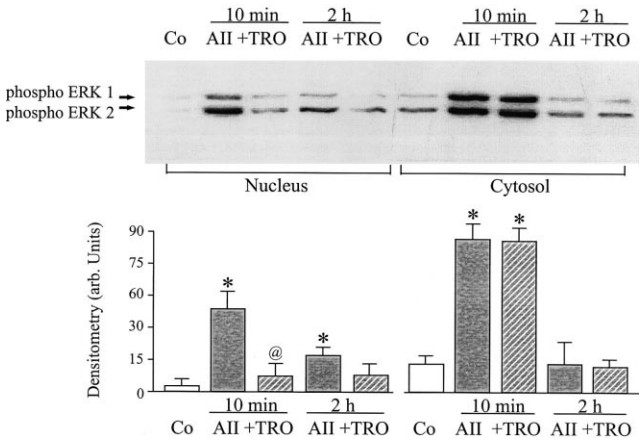

Fig. 2. TRO inhibits the activation of nuclear MAPK, but not its cytosolic activation by AII. Quiescent VSMCs were incubated with TRO (10 μM) for 30 min before AII (1 μM) was added. Cells were then stimulated with AII for 10 min or 2 h, lysed and equal amounts of protein (30 μg) of nuclear and cytosolic cell extracts were separated on 7.5% SDS-PAGE gels. Immunoblotting was performed with a phospho-specific ERK1/ERK2 MAPK antibody. The Western blots shown are representatives of three experiments using different cell preparations. The densitometric data are expressed as mean ± S.E.M., * = $P < 0.05$ versus control, @ = $P < 0.05$ versus AII alone for 10 min.

ERK 1/2 in VSMCs, as it has been described in other cell types, and whether TRO interferes with this process.

Nuclear translocation of ERK 1/2 was observed 10 min after stimulation with AII (8 ± 2.5-fold over control, $P < 0.05$), with a further accumulation of total nuclear ERK 1/2 protein after 2 h (15.5 ± 4-fold over control, $P < 0.05$) (Fig. 3A). These data are consistent with reports on the time-course of nuclear ERK 1/2 translocation in other cell types [30,31]. The nuclear translocation of ERK 1/2 was accompanied by a concomittant decrease in the amount of cytosolic total ERK 1/2 protein at the 2 h time-point.

TRO completely inhibited the nuclear translocation of

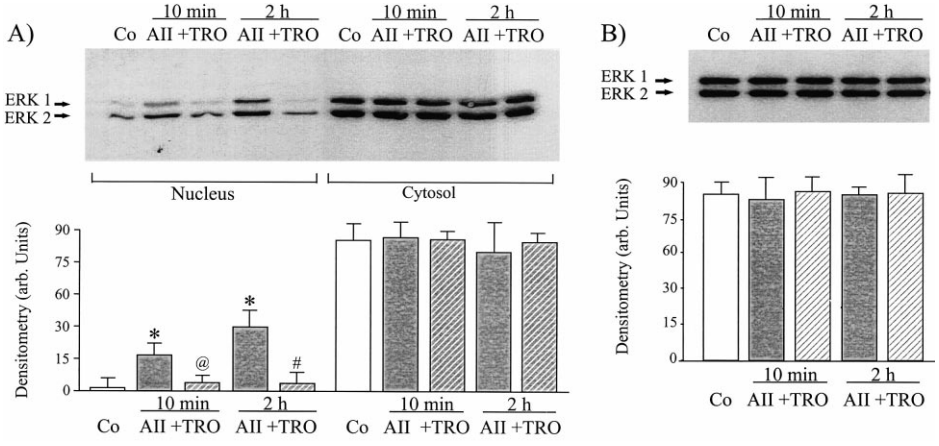

Fig. 3. TRO inhibits AII-induced nuclear translocation of ERK 1/2 in VSMCs, without affecting total ERK 1/2 protein levels in whole cell extracts. (A) Cell treatment and sample preparation was performed as described in Fig. 2. Nuclear and cytosolic extracts were separated on 7.5% SDS-PAGE gels, followed by immunoblotting with an antibody against total ERK1/ERK2 ERK 1/2. (B) Quiescent VSMCs were incubated with TRO (10 μM) for 30 min prior to the addition of AII (1 μM). Cells were then incubated for 10 min or 2 h, lysed and whole cell extracts were prepared. Equal amounts of protein (30 μg) were separated on 7.5% SDS-PAGE gels and immunoblotted against total ERK1/ERK2 MAPK. The Western blots shown are representative for three experiments using different cell preparations. The densitometric data are expressed as mean ± S.E.M., * = $P < 0.05$ versus control, @ = $P < 0.05$ versus AII alone for 10 min, # = $P < 0.05$ versus AII alone for 2 h.

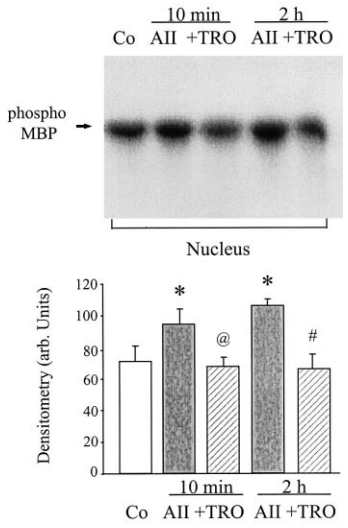

Fig. 4. TRO inhibits AII-stimulated nuclear PKCζ activity. Quiescent VSMCs were treated as described in Fig. 1 and nuclear extracts were prepared. After immunoprecipitation of equal amounts of protein (50 μg) with a PKCζ antibody, kinase assays for PKCζ activity were performed. Samples were then separated on 12% SDS-PAGE gels and phosphorylation of MBP (phospho-MBP) by PKCζ was determined by autoradiography. The autoradiogram shown is representative for three experiments using different cell preparations. Results of the densitometric analysis are depicted in the lower panel. Data are expressed as mean ± S.E.M., * = $P < 0.05$ versus control, @ = $P < 0.05$ versus AII alone for 10 min, # = $P < 0.05$ versus AII alone for 2 h.

ERK 1/2 (Fig. 3A), without affecting the amount of ERK 1/2 protein in whole cell extracts (Fig. 3B). These results demonstrate that the observed inhibitory effect of TRO is due to the blockade of nuclear translocation, but not downregulation of ERK 1/2 protein levels.

3.4. TRO inhibits AII-stimulated nuclear PKCζ activity

PKCζ has been shown to translocate into the nucleus after growth factor stimulation, where it phosphorylates nuclear targets such as nucleolin [12–15]. PKCζ, therefore, may function as a potential upstream signaling kinase for AII-stimulated ERK 1/2 activity in both the cytoplasm and the nucleus. In order to investigate whether TRO inhibits not only the nuclear translocation of ERK 1/2 but possibly also the nuclear activation of ERK 1/2 by AII-induced PKCζ, we examined the effects of TRO on PKCζ activity in the nuclear fraction.

Examining PKCζ activity with an immune complex phosphorylation assay, we found that PKCζ is constitutively active in the nucleus, as evidenced by the bands detected in unstimulated controls (Fig. 4). This is consistent with previous observations of constitutive PKCζ kinase activity in PKCζ RNA micro-injected oocytes [32] or insect cells expressing rat PKCζ [33]. Stimulation with AII caused an increase in the nuclear PKCζ activity at 10 min and 2 h by 1.31-fold and 1.47-fold, that was completely blocked by TRO (all $P < 0.05$) (Fig. 4). The inhibition of PKCζ activity in the nuclear compartment by TRO, however, was not related to an inhibition of the PKCζ movement from the cytoplasm to the nucleus, as was the case for ERK 1/2 trafficking. As shown in Fig. 5, PKCζ is present in the nuclear and cytosolic compartment of unstimulated cells and does not translocate upon stimulation with

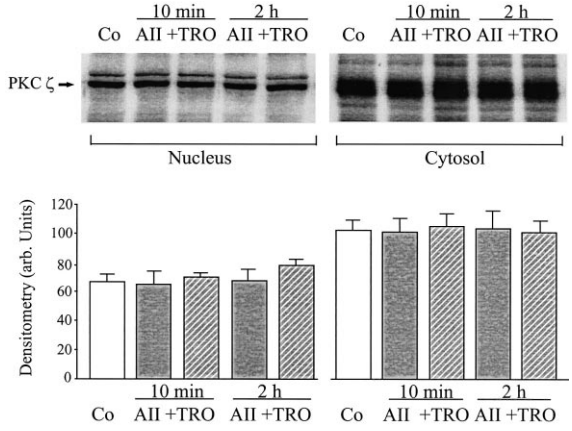

Fig. 5. Total PKCζ protein is not affected by TRO and does not translocate to the nucleus after AII stimulation. Cell treatment and sample preparation were performed as described in Fig. 2. Nuclear and cytosolic extracts were separated on 7.5% SDS-PAGE gels, followed by immunoblotting with an antibody against PKCζ. The Western blots shown are representative of three independently performed experiments and the results of the densitometric analysis are shown as mean ± S.E.M.

AII. Treatment with TRO had no effect on the amount of total cytosolic or nuclear PKCζ protein.

These results indicate that TRO inhibits both AII-induced nuclear translocation of ERK 1/2 and the nuclear activity of its upstream signaling kinase, PKCζ.

4. Discussion

The present investigation demonstrates that TRO inhibits AII-induced nuclear translocation of ERK 1/2 and the kinase activity of PKCζ in the nucleus. The ERK1/2 MAPKs are serine/threonine protein kinases which are required for the mitogenic and chemotactic effects of AII in VSMCs [3]. Nuclear translocation of activated ERK 1/2 is a critical event for the ERK 1/2-dependent regulation of transcription factors and other signaling molecules in the nucleus that modulate cell behavior [18,19]. PKCζ functions not only as an upstream cytosolic kinase for AII-induced activation of the MEK/ERK 1/2 pathway [11], but can phosphorylate and activate nuclear targets as well [15]. Thus, both kinases may be important for regulating VSMC proliferation and migration by interacting at cytosolic and nuclear levels to regulate the transduction of AII-induced signals into the nucleus.

Our data reveal that TRO inhibits AII-induced ERK 1/2-dependent signaling in VSMCs through effects on multiple targets. These findings may explain our previous observations that TRO inhibited bFGF and PDGF signaling pathways in VSMCs which regulate ERK 1/2-dependent cell functions, but had no effect on the ERK 1/2 activity induced by these growth factors [6]. Similar to our findings in this study, the effect of TRO on PDGF and bFGF signaling may be due to an inhibition of ERK 1/2 translocation into the nucleus, while the total ERK 1/2 activity in response PDGF or bFGF signaling through their tyrosine kinase receptors is unaffected.

The partial inhibition of AII-induced ERK 1/2 activation by TRO is likely due to its inhibition of the AII-stimulated nuclear PKCζ activity. Whereas TRO did not affect the cytosolic PKCζ activity and the consequent activation of cytosolic ERK 1/2 after AII stimulation, it markedly inhibited the AII-

induced nuclear PKCζ activity. Although it has been reported that PKCζ can undergo nuclear translocation upon growth factor stimulation [12–14], we did not observe a movement of PKCζ protein from the cytosol to the nucleus after stimulation with AII. In our experimental conditions, PKCζ was expressed and constitutively active in both the cytosolic and nuclear compartment, which is consistent with reports on other cell types [14,32–34]. Thus, unlike the effect of TRO on ERK 1/2 trafficking, the inhibition of nuclear PKCζ activity is not due to an inhibition of translocation. PKCζ is known to activate MEK [11,28,29], which in turn leads to ERK 1/2 activation. Although there is no conclusive evidence that this signaling cascade functions in the nucleus, mitogenic stimulation of COS7 cells leads to a rapid nuclear translocation of MEK [35]. Taken together, these data suggest a role for PKCζ in activating the nuclear ERK 1/2 protein, which could explain the partial inhibition of AII-induced ERK 1/2 activation by TRO in whole cell extracts, that we have previously reported [5].

Our finding, that TRO inhibits the nuclear translocation of ERK 1/2 and nuclear PKCζ activity in response to AII, thereby ultimately leading to an inhibition of nuclear ERK 1/2 activation, provides strong evidence that TRO specifically targets nuclear ERK 1/2 signaling steps. A nuclear site of action of TRO, however, is not unexpected since TRO is a known ligand for PPARγ, a nuclear transcription factor of the steroid hormone receptor superfamily [24,25]. Since PPARγ is mainly localized in the nucleus of VSMCs [36], this receptor likely mediates the nuclear effects of TRO. Inhibition of signaling through PPARγ is also consistent with our data showing that TRO had no effect on AII-induced cytosolic PKCζ activation or ERK 1/2 activity. A possible mechanism for an inhibition of ERK 1/2 translocation by PPARγ could be due to protein-protein interactions, as it has been shown for PPARγ-related transrepression of the transcription factors NFkB and AP1 in macrophages [37].

The inhibition of ERK 1/2-mediated phosphorylation of nuclear proteins by TRO could constitute the mechanism by which this agent blocks AII-induced VSMC growth and migration. This hypothesis is in line with the observed ability of TRO to inhibit signaling through multiple growth factor pathways, which all converge at the activation of ERK 1/2 [5,23] and may explain how TRO suppresses vascular lesion formation [6]. We and others have shown that TRO inhibits neointima formation after balloon injury in non-diabetic [6] and diabetic rats [38]. Similar vascular protective effects have been described for ACE inhibitors and AT1 receptor blockers in a comparable rat model [1]. Moreover, a recent study emphasizes the importance of AII-induced ERK 1/2 activation in vivo, where the rapid activation of ERK 1/2 in the rat aorta after balloon injury was inhibited by a AT1 receptor blockade and ACE inhibition [39]. However, clinical trials using ACE inhibitors failed to prevent restenosis after coronary angioplasty in man [40,41]. These contrary results may be due, in part, to the additional effects of other growth factors involved in vascular remodeling. By targeting nuclear ERK 1/2 translocation and activation, TRO may inhibit the critical signaling steps common to the action of multiple growth factors, thereby limiting the development and progression of restenotic and atherosclerotic lesions.

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